

This is a repository copy of *High-Throughput, low-loss, low-cost, and label-free cell separation using electrophysiology-Activated cell enrichment*.

White Rose Research Online URL for this paper:

<https://eprints.whiterose.ac.uk/115176/>

Version: Accepted Version

Article:

Faraghat, Shabnam A., Hoettges, Kai F., Steinbach, Max K. et al. (5 more authors) (2017) High-Throughput, low-loss, low-cost, and label-free cell separation using electrophysiology-Activated cell enrichment. *Proceedings of the National Academy of Sciences of the United States of America*. pp. 4591-4596. ISSN 1091-6490

<https://doi.org/10.1073/pnas.1700773114>

Reuse

Items deposited in White Rose Research Online are protected by copyright, with all rights reserved unless indicated otherwise. They may be downloaded and/or printed for private study, or other acts as permitted by national copyright laws. The publisher or other rights holders may allow further reproduction and re-use of the full text version. This is indicated by the licence information on the White Rose Research Online record for the item.

Takedown

If you consider content in White Rose Research Online to be in breach of UK law, please notify us by emailing eprints@whiterose.ac.uk including the URL of the record and the reason for the withdrawal request.

High-Throughput, Low-Loss, Low-Cost and Label-Free Cell Separation using Electrophysiology Activated Cell Enrichment (EPACE)

Shabnam A. Faraghat¹, Kai F. Hoettges¹⁺, Max K. Steinbach^{1,2}, Daan van der Veen³, William J. Brackenbury⁴, Erin A. Henslee¹, Fatima H. Labeed¹, Michael P. Hughes^{1*}

¹Centre for Biomedical Engineering, Department of Mechanical Engineering Sciences, University of Surrey, Guildford, Surrey GU2 7XH, United Kingdom

²Hochschule Fresenius, Limburger Str. 2, 65510 Idstein, Germany

³School of Biosciences, University of Surrey, Guildford, Surrey GU2 7XH, United Kingdom

⁴Department of Biology, University of York, Heslington, York, YO10 5DD, UK

+Present address: Department of Electronic and Electrical Engineering, University of Liverpool, Merseyside L69 3BX

*Corresponding author. Email m.hughes@surrey.ac.uk, tel +44 1483 686775

Classification

Physical sciences/applied physical sciences

Biological sciences/applied biological sciences

Key words

MACS, FACS, dielectrophoresis, dielectrophoretic, DEP

Abstract. Currently, cell separation occurs almost exclusively by density gradient methods and by fluorescence- and magnetic-activated cell sorting (FACS/MACS). These variously suffer from lack of specificity, high cell loss, use of labels, and high capital/operating cost. We present a dielectrophoresis (DEP)-based cell separation method, using 3D electrodes on a low-cost disposable chip; one cell type is allowed to pass through the chip whilst the other is retained and subsequently recovered. The method advances usability and throughput of DEP separation by orders of magnitude in throughput, efficiency, purity, recovery (cells arriving in the correct output fraction), cell losses (those which are unaccounted for at the end of the separation) and cost. The system was evaluated using three example separations; live and dead yeast; human cancer cells/red blood cells; and rodent fibroblasts/red blood cells. A single-pass protocol can enrich cells with cell recovery of up to 91.3% at over 300,000 cells/second with >3% cell loss. A two-pass protocol can process 300,000,000 cells in under 30 minutes, with cell recovery of up to 96.4% and cell losses below 5%, an effective processing rate >160,000 cells/second. A three-step protocol is shown to be effective for removal of 99.1% of RBCs spiked with 1% cancer cells, whilst maintaining a processing rate of ~170,000 cells/second. Furthermore, the self-contained and low-cost nature of the separator device means that it has potential application in low-contamination applications such as cell therapies, where GMP compatibility is of paramount importance.

Significance statement. Cell separation is a fundamental process in biomedicine, but is presently complicated, cumbersome and expensive. We present a technique that can sort cells at a rate equivalent to or faster than gold-standard techniques such as FACS and MACs, but can do label-free and with very low cell loss. The system uses dielectrophoresis (DEP) to sort cells electrostatically, using a novel electrode chip that eschews microfabrication in favour of a laminate drilled with 397 electrode-bearing wells. This high level of

parallelisation makes the system immune to the bubbles that limit labs-on-chip, whilst also increasing capacity and throughput to unprecedented levels, whilst the chip is cheap enough to be disposable, preventing inter-separation contamination.

/body

1. Introduction

Since the separation of red and white blood cells was first reported in 1974 (1), the technology has become fundamental in the biomedical sciences. From isolation of diseased tissue to the identification of cells for therapeutic uses and the potential for regenerative cell based therapies, cell separation techniques are increasingly integrated into other fields such as biochemistry, electrical engineering, physics and materials science (2). Three methods dominate cell separation. Density gradient methods exploits differences in density between populations such as red/white blood cells. Fluorescently-activated cell sorting (FACS) uses fluorophore-conjugated antibodies as a discriminator; cells are launched in droplets, each containing one cell, through a fluorescence detection system to determine the cell type and are then electrostatically diverted into different output receptacles (3). FACS can sort up to 50,000 cells per second, with higher rates achievable at the cost of purity. Finally, magnetically-activated cell sorting (MACS) uses magnetic microbeads conjugated with antibodies. These bind to targets on cell surfaces, which then can be extracted by applying a magnetic field (4).

However, the three methods have drawbacks. Only density gradient does not require the use of chemical labels; the others use fluorescent chemicals or antibodies to indicate the target population. These are expensive and may have limited specificity; in the case of MACS, the target protein *must* be present on the surface of the cells. Following separation, the labels may also persist in the cells, limiting their usefulness. Cell losses in FACS and MACS can exceed half the population, particularly at high sorting rates (5,6). FACS machines (particularly high-throughput devices) are very expensive and are not easily cleaned, rendering cell populations separated by this method non-compliant with good manufacturing practice (GMP). Finally, as the cell preparation includes a period during which labels are added and

then given sufficient time to conjugate, the total time to perform the sort can extend over several hours.

There have been many attempts to develop cell separation techniques that are affordable to buy and use, do not require labels, and are able to retain significant numbers of cells. An alternative separation technique is dielectrophoresis (DEP). A polarisable particle suspended in a non-uniform electric field (7) interacts with the field gradient, inducing a dipole. The interaction of dipole and field gradient produces different Coulomb force on either pole, inducing motion up or down the high field gradient according to the polarity of the dipole. The magnitude and polarity of the dipole itself is governed by the electrical properties of the cell (resistance and capacitance of membrane and cytoplasm) and medium, and the frequency of the electric field. DEP can be used both to characterize and separate cells according to the passive electrical properties; where two cell types experience a field gradient at a frequency such that one cell type is attracted to the electrodes, one repelled. The repelled cells pass through the device unaffected, but the others are attracted to, and retained by, the electrodes acting as an “electrostatic filter”. When the field is removed, these cells are released and can be collected separately. For example, Figure 1 shows spectra of two identical cell types, save that one has double the membrane capacitance of the other. The highlighted frequency range indicates where one cell types experiences positive DEP, the other negative. DEP spectra can be determined by commercial devices such as the DEPtech 3DEP, and exploitable differences can be readily identified by visual inspection.

DEP separation of live and dead yeast cells was first demonstrated in 1966 (8). Subsequent research demonstrated separation of cancerous and healthy cells, gram-negative and gram-positive bacteria, stem cell subpopulations, different types of viruses, of for sample preparation whilst differences between cells indicated the potential for DEP to fraction leukocyte subpopulations (9-20). However, few approaches to DEP have been able to

compete with MACS or FACS; implementations often suffer from cells being trapped in interconnecting tubing, devices requiring small chamber heights to ensure cells pass close to the electrodes, restricting throughput and ultimately limiting total cell capacity; and the high cost of manufacturing, making it difficult to mass-produce the separator as a reliable, disposable component. There are exceptions; the ApoStream method (21) can isolate significant numbers of circulating tumor cells (CTCs) from 12 million nucleated peripheral blood cells in 60 minutes ($5,000 \text{ cells s}^{-1}$). Hu et al. (22) reported separating 10,000 cells per second, but required the use of chemical labelling. Lee et al (23) reported a rate of 616 cells s^{-1} . Markx et al. (24) processed live and dead yeast cells at $10^7 \text{ cells ml}^{-1}$ with purity in excess so 93%; however, the total capacity of the device was only $50 \mu\text{l}$, limiting overall throughput. An alternative approach to DEP separation used 3D chips constructed from interleaved conducting and insulating sheets with holes or “wells” drilled through (25). The first prototype sorted 50:50 live and dead yeast to 86:14 at 0.4 ml min^{-1} . A subsequent design (26) included remixing by passing a through multiple wells, producing high cell recovery (>90) but low throughput ($25 \mu\text{l min}^{-1}$).

To be comparable to FACS or MACS requires cell sorting at rates in excess of 10^5 s^{-1} with minimal cell loss, high purity, low cost and highly robust. Here we describe an electrophysiology-activated cell enrichment (EPACE) method employing a chip with 397 holes of $400 \mu\text{m}$ diameter drilled through a laminate of 12 conducting layers separated by insulators, though which 397 wells with $400 \mu\text{m}$ diameter were drilled. As shown in figure 2, this creates 397 parallel paths through the chip, each with 12 electrodes along the bore. This high degree of parallelization, and the fact that all cells are no more than $200 \mu\text{m}$ from the electrodes, allows very high cell processing rates whilst minimising cell loss, and creates immunity from bubbles (which will, at worst, block only one channel). Consequently, the system can separate cells at rates substantially in excess of $320,000 \text{ cells s}^{-1}$ measured across

all cell types studied, separating populations in excess of 10^8 cells into output receptacles in under 30 minutes with cell losses as low as 0.3%, and separation efficiencies as high as 96.4%, achieving more than 30 times higher throughput of previous DEP devices.

2. Results

2.1. Cell enrichment using a single-pass protocol.

To assess separation efficacy, binary mixtures of cells were processed through the device in 5-15ml units, processed at between 15,000 – 33,000 cells s^{-1} , the results of which are summarised in Figure 3. Live and dead yeast showed mean recovery (the proportion of cells output in the correct outlet) of 81.3% and 89.5% respectively; vole blood and fibroblasts showed separation efficiencies of 87.3% and 73.9% respectively. Purities (the proportion of required cells in the output population) at the output for the above populations were 91.3% and 78.2%, for live and dead yeast, and 75.6% and 86.4% for vole RBCs and fibroblasts. Whilst these values are low when considering the technique as a separation *per se*, they represent sufficiently high values to constitute effective cell enrichment. It is also significant that for the above population sorts, the mean cell losses (cells which were input but are unaccounted for at the output, due to damage, adherence to the electrodes or device casing; or being left in residual liquid in the device at the end of the experiment) were 1.4%, 2.7%, 4%, and 0.3%, respectively. These low cell losses are important for two reasons; firstly, multiple passes can be used to improve performance without significantly degrading cell numbers; secondly, unlike methods such as FACS where a significant number of cells being separated are destroyed by the separation process (6), this protocol does not reduce cell number – an important issue where initial cell numbers are low. Experiments at 0.5 and 0.8 and 1.0 ml min^{-1} yielded similar results, suggesting these metrics represented a plateau of trapping efficiency, and that the device was working well within its performance limit.

To assess the viability of cells post-sort, collected fibroblasts were suspended in fibroblast growing medium, divided into two T75 flasks and placed in the incubator to assess growth potential. The cells were observed 1 and 3 days after incubation and were found to have adhered to the flask and reached confluency respectively. A DEP spectrum of the cells was obtained and a further passage performed to check for abnormalities and none were observed.

In order to identify the upper throughput limit, live yeast cells were captured in the device in increasing concentrations, as it is the fraction of cells experiencing positive DEP that are limited by the capacity and efficacy of the DEP electrodes (the other, repelled, population simply passing through the device unaffected). Retaining the 1 ml min^{-1} flow rate, the concentration of cells per ml was increased from 10^6 ml^{-1} to $\sim 2 \times 10^6$, 5×10^6 , 10^7 , 2×10^7 and $10^8\text{ cells ml}^{-1}$. Chip saturation was monitored in two ways: firstly, we measured the total number of cells retained in the chip and subsequently recovered. Secondly, cells were aliquoted in 1ml fractions and analysed to determine the cell ratio in order to observe trap saturation. Saturation can then be detected by the appearance of a higher proportion of live cells in the output stream. Any saturation effect would only affect cells collected by positive DEP on the electrodes; cells experiencing zero or negative DEP pass through the chip unimpeded and are collected in the receptacle below, and as a consequence there is no saturation limit on such cells.

At concentrations up to $2.7 \times 10^7\text{ ml}^{-1}$ the device worked as for $1 \times 10^6\text{ ml}^{-1}$. When concentrations of $2.7 \times 10^7\text{ ml}^{-1}$ were used, the aliquots were normal and the mean cell recovery was 279 ± 12.2 million cells. When the input concentration was $5.9 \times 10^7\text{ ml}^{-1}$, the number of cells increased dramatically (by over 300%) at the 6th aliquot, and the mean total of recovered cells was 321 ± 9.0 million cells; when $1.07 \times 10^8\text{ ml}^{-1}$ were used, the device became saturated after the 3rd aliquot (rising by 38% in aliquot 4, rising to >100% in aliquot 7 and later), and recovery indicated that the trap contained $359 \pm 13.3 \times 10^6$ cells. After the

separation run was completed, the cells collected in the chip were flushed and counted. In the three instances where the chip was saturated, the number of cells retained before the chip was saturated, with upper limits of approximately 350×10^6 cells representing the capacity of the chip.

The difference in capacity between 59 million and 109 million cells/ml is interesting, as it suggests the trap capacity may be increased at high concentrations due to cell-cell interactions; however, it appears clear that a 300×10^6 cell sort (20 million cells/ml, 15 ml total volume) represents the maximum effective cell separation where the cell content is entirely unknown, though this represents the limit only on the positive DEP fraction; a sort of 300 million cells experiencing positive DEP from a further 300×10^6 (or more) experiencing negative DEP would in principle be entirely workable, raising the throughput past 0.6×10^9 cells/ml.

2.2. High-volume separation performance using multi-pass protocols

Whilst enrichment on this scale is of interest, a second or third pass through the device enables the user to collect cells that were not retained in previous passes, significantly improving performance in a very short time. There are two strategies for multiple sorting passes; re-sorting the effluxed and retained cells, respectively. Here we examine these two protocols separately; a two-pass strategy re-processing the effluxed cells to minimise cell loss whilst maximising enrichment in the shortest possible time, and a three-pass strategy where the collected cells are re-processed.

Enhancing the negative DEP population. We sorted mixtures of $9.6 - 14 \times 10^6$ cells (similar to the cell number in a confluent T175 flask) at concentrations of $1-2 \times 10^6$ cells ml^{-1} and passed through the EPACE twice. After each run, the chip was flushed with fresh medium to recover cells retained by positive DEP before the output was reprocessed.

Negative DEP was used to select the dead cells from mixtures of live and dead yeast cells, or RBCs from mixtures of RBCs and fibroblasts, the results of which can be seen in Figure 4. After the second pass the purity of dead yeast was increased to 93.4%; whilst the RBC fraction increased to 93.8%. However, recovery was reduced, falling to 81% and 73.7% respectively, though these are still substantially above recovery rates for FACS and MACS. Overall cell losses (cells not appearing in either outlet) were 2.7% and 4%, with the remaining cells appearing in the other (waste) output. We postulated that the values of separation efficiency for the populations experiencing negative DEP were adversely affected by cells being trapped in the dead volume between the chip and outlet, which were then recovered with the retained cells rather than passing to the effluxed portion. In order to verify this we performed a separation of 20ml of RBC/fibroblast cells at the same concentration. After the second pass, the recovery of RBCs was broadly similar at 87.8%, but the purity raised to 96.6%.

Enhancing the positive DEP population. An important application of cell separation is the enrichment of relatively rare subpopulations. In order to use the EPACE platform to enrich highly asymmetrical cell concentrations a 3-pass strategy was used, with the cell fraction retained in the electrodes being retrieved and then subject to two further iterations (total time taken ca. 30 minutes). The cells collected were counted after each pass, and the ratio of cell types was determined. These results are summarised in Figure 5. Human red blood cells were spiked with MDA-MB-231 breast cancer cells, to a final concentration of 1.1% cancer to 98.9% RBCs, at a total cell concentration of ca. $2 \times 10^7 \text{ ml}^{-1}$, to a total sample volume of 4ml. Cells were passed through the device at 0.5 ml min^{-1} . After the cells mixture had completed its first pass through the device, the fraction retained in the device was released in 1ml of fresh medium which was topped up to 3 ml with fresh medium after cell counting. Sampling indicated that the concentration of RBCs had dropped to 95.5% of the output. The

3 ml of solution was then subject to a second pass and resuspension, at which point RBCs had dropped to 75.6%; and after the third pass, RBCs made up 59.5% of the cell sample, with cancer cells forming the remaining 40.5% of the cells, an effective removal rate of the RBCs of 99.1%. At the end of the process, on average 47.7% of the initial population of cancer cells were recovered. However, those that had not been collected in the initial sort would be available for re-sorting in the waste from the previous passes, which could be used to significantly increase cell recovery if required, by adding an additional pass on the eluted cells.

3. Discussion

3.1 Analysis of performance.

Whilst purity of sample at output is the final arbiter of the quality of a separation/enrichment method, it is difficult to use as a guide as it depends on the concentration of the two cell types at the input. Instead, we can look at the concentration of both the desired and undesired cells. Analysing the 2-pass protocol to enrich live dead yeast and RBC/fibroblast cells by negative dielectrophoresis, the population experiencing negative DEP has recovery of approximately 90% for the desired cells and 20% for the undesired fraction for each round of enrichment. After two passes, these are approximately squared, such that approximately 0.81x the desired and 0.04 undesired cells are present at the output, leading to a purity of $0.81/(0.81+0.04) = 95.3\%$, in line with experimental results. Similarly, for enrichment of cells experiencing positive dielectrophoresis, across the three rounds of separation the same process was observed, with an average of approximately 80% of MDA cells and 20% of RBCs appearing at the output for each round of enrichment. Over successive passes, this predicts RBC proportions of 96%, 86% and 60% in line with observations. The cell losses are sufficiently low for them to be disregarded in the calculation.

3.2 Effect of Design on Optimum Performance

From our results, it is possible to identify ways to optimise the cartridge design and increase separator performance by improving the values of cell recovery for the wanted and unwanted cells. Notably, whilst the peak values of recovery and purity are high, it is the passed cells that have the highest purity, whilst retained cells always have the highest recovery. As configured, the device contains two “dead volumes” of sample that cannot be recovered at the end of the experiment. One occurs due to the use of a conical syringe plunger; at maximum insertion the tip of the cone touches the chip, 0.56 ml of unseparated cells remain surrounding the plunger tip. Similarly, approximately 0.54 ml of post-separation cells remain in the space between the chip and outlet. Consider a mixture of two populations A and B, where population A experiences negative DEP and passes through the chip whilst population B is retained by positive DEP; the mixture is contained in a 10ml sample. After the solution is passed through, 1.1ml (11%) of cells remain in the dead volume, limiting maximum recovery of A to 89%. However, purity is unaffected because only the cells which have been passed through by the chip are collected. When the solution has been processed, we then draw 1ml into the device and release population B. However, the device also contains 1.2ml of the original cell mixture, containing cells from population A. This means that the recovery rate for mixture B is as high as can be obtained (since all of the cells are in the extracted volume), but the purity is downgraded by the presence of population A cells in the dead volumes. In effect, the dead volumes act to take cell mixtures intended for the output for population A, and deposit them in the output to population B. Whilst a future embodiment of the device could reduce the dead volumes by redesigning the plunger and outlet path, we can calculate the effective peak separation by mathematically removing the cells in the dead volumes. We estimate that using this approach, the recovery of RBCs and purity of fibroblasts in the separation described above would equal or exceed the same parameters for the two

parameters unaffected by the dead volumes, which is to say that all purities and recoveries would exceed 95% for a two-pass strategy.

The design outlined in this paper is a proof of concept, but a number of design modifications suggest themselves to improve simplicity, throughput, and capacity. For example, the device presented here is loaded manually by drawing the syringe plunger upwards. This means there is little control of the flow rate during loading; hence, during this time, the chip is not energised. However with the application of a bidirectional syringe pump that can both push and pull, it would be possible to separate on both draw and expel cycles, making separation simpler and more efficient. The single inlet of the cartridge also lends itself to combining with simple liquid handling /fraction collection systems, allowing automation of complex multi-pass protocols without user intervention. It is also evident that the design presented here could be modified to increase throughput. For example a second chip, independently energised, could be mounted below the first to permit a second stage of separation; this could either be at the same frequency as the first, doubling the separation rate by removing the necessity for two separation passes, or at a different frequency to allow two-parameter sorting, whilst requiring no further antibodies (as would be required for FACS and MACS), making for a negligible cost increase over a single-chip version. Similarly the throughput and total cell capacity could be increased by enlarging the chip and increasing the number of wells; this could be achieved by making a single electrode disk larger, or otherwise increasing the number of wells. For example, a chip with a ca. 3500 wells could potentially separate up to 10^{10} cells in a similar 30 minute period, potentially allowing separation on industrial scales, or clinical applications such as sorting of stem cells from bone marrow.

3.3. Comparison to other separation methods

As described earlier, cell separation methods requiring more sensitivity than density gradients can be addressed by two methods; FACS and MACS. A high throughput FACS system is

expensive; both FACS and MACS require expensive labels, and can only separate cells on the basis of this labelling. The EPACE system presented here separates on the basis of physical parameters without the need for labels, and both the instrumentation (pump and generator) and consumables required (media, chips) are an order of magnitude less expensive than either method. Furthermore, the system presented here could be manufactured as a fully assembled, sterile, integrated cartridge; with no crossover possible with other separation runs, and no residual chemical labels to adversely affect cells, this allows the cells produced to be fully compliant with Good Manufacturing Practice (GMP).

Cell throughput rates also compare well with other methods. High throughput FACS cell sorters can process up to 100,000 events per second, though a relatively small proportion of these may actually contain cells. MACS is a bulk method rather than processing cells serially, but the time taken to prepare and perform separation is comparable to the method presented in this paper. Similarly, the number of cells to be separated by high volume MACS systems is 10^9 cells; whilst no upper limit for FACS has been identified, a wide review of the literature report few studies exceeding 2×10^7 cells; baseline EPACE performance is comparable to MACS, and better than FACS. For example, the capacity of the device is approximately 4×10^8 yeast cells; balancing the relatively small size (and hence higher packing density) of such cells against the fact that DEP trapping force scales with cell volume, a capacity of $>10^8$ mammalian cells is certainly not unreasonable. In terms of throughput, if we divide the number of cells processed by the time required for a two-pass sort, we have an effective sorting rate of 167,000 cells per second; adding in a third sort, as in the case of the rare cell study, still gives a nett throughput comparable to high-throughput FACS. Considering cell loss, EPACE is shown to be comparable to, or better than, either existing method. In no case were more than 7% of cells lost, and in many cases fewer than 3% of cells were lost. By comparison, FACS typically loses $>50\%$ of cells through cell

damage during droplet formation or rejection due to incorrect scanning, particularly at higher flow rates (5). When used in three-pass mode, cell recovery was lower, but the cells not initially captured by the device will be available for re-sorting in the outlet stream by using a more complex multiple-run separation strategy.

Comparing our method to published DEP methods, the EPACE process is 30x faster than the Hu et al. (22) system, 6x faster than the Gupta et al. (21) system, and 600x faster than the system presented by Lee (23), with comparable or better cell recovery and cell losses. The cell concentration and recovery were comparable to the Marx et al. system (24); without additional data we cannot compare throughput or cell loss. As these devices were microfluidic, we suggest that the system presented here should be significantly more robust; whereas the function of planar microfluidic devices can be compromised by the presence of a single bubble, the highly parallel design of our chip means that if a bubble appears it has no more effect potentially blocking just one of the 397 holes.

Unlike FACS and MACS, which sort cells on the basis of specific recognition of membrane proteins, DEP separates on the basis of differences in the electrophysiology of the two cell types. This offers advantages over these existing methods, as many differences in cell types (and changes in cells in response to external stimuli) exhibit corresponding differences in the cell's electrophysiology. Examples have included the differentiation of neural stem cells, where electrical changes in the membrane allow sorting of cells according to differentiation fate far in advance of conventional marker-based methods e.g. (27- 29); indeed, stem cell sorting may be an area where DEP-based separation may dominate over existing methods, whilst DEP separations in cancer cells have been reported for some decades (13), both for diagnosis and development of interventions. Many other cases of differential DEP response have been identified in the past 50 years, and all past demonstrations can be performed using this platform (30). Once differential electrophysiology is established by DEP profiling, it can

be exploited through the use of ion channel blockers to emphasise difference and enhance DEP separation (31).

In conclusion, we have presented a DEP-based cell separation technique that has a capacity and throughput comparable to the fastest MACS and FACS machines, requires no chemical labels, offers GMP compatibility, significantly lower cell loss, and significantly lower capital and running costs. Given the opportunity to exploit differences in cell electrophysiology in fields such as stem cell therapy and cancer, we believe this offers significant promise as a new standard benchtop laboratory technique.

Materials and Methods

Experiments followed the steps outlined in figure 6. The cartridge and chip were cleaned with ethanol before assembly. The chip manufacture and cell mixtures are described in the Supplementary Material. A sample containing a mixture of the two cell types A and B was loaded into the fluidic cartridge by inserting the tube into the solution, then manually withdrawing the plunger to load the reservoir. This was then placed in a vertically-mounted syringe pump (Razel, Stamford, USA; maximum output of 1 ml/min), and the solution was pumped through the chip whilst voltage was applied to the electrodes using signal generator (Jupiter 2000, Blackstar, St Ives, UK), connected to the chip via a custom-made amplifier board to supply up to 18V_{pp} at frequencies up to 1MHz. The output (enriched for population A) was collected in a second receptacle. Then fresh solution was loaded into the cartridge, the field was deactivated, and the chip was flushed manually with fresh medium to dislodge and recover the cells from population B that had been collected by positive DEP. When using a second pass or third pass, it was possible to enrich either cell-A or cell-B samples by repeating the procedure with the enriched samples. The total time for the two-pass procedure was under 30 minutes for yeast, and under 15 minutes for RBC/fibroblasts. Cells were

exposed to the electric field for an average of approximately 5 minutes for the positive DEP fraction, or under 10 seconds for the negative DEP fraction. All experiments were repeated three times. For the three-pass experiments to enrich rare cells, at the end of the second and third pass the cells were recovered by switching the field off, manually pulling the plunger up slightly aspirating air into the fluidic cartridge and expelling the retained cells in the small sample volume that is still within the chip. Separations were evaluated for cell recovery (the proportion of desired cells which arrive at the correct outlet), purity (the proportion of desired cells in a given outlet) and cell loss (the number of cells which are unaccounted for at the end of the separation). The number of missing cells was obtained by subtracting the ‘passed’ and ‘recovered’ populations from the initial number of cells.

DEP spectra of individual cell types were measured using a DEPtech 3DEP (Uckfield, UK) and a separation frequency midway between the crossover frequencies (the frequency on the DEP spectrum where the response crosses zero) for the two cell types to be separated; for the live/dead yeast mixture, 1 MHz was selected; for the fibroblast/RBC mixture, 22 kHz was selected; and for the MDA/RBC mixture, 76 kHz was used. Cells in output mixtures were either identified by the use of Trypan blue (live and dead yeast) or by visual inspection of the morphologically quite different RBCs and fibroblasts/MDAs.

Acknowledgements

We wish to thank Mr David Gould and Mr Rowan Lonsdale for their assistance in constructing the system, and DEPtech for funding the manufacture of the chips.

REFERENCES

1. English D, Andersen BR (1974) Single-step separation of red blood cells, granulocytes and mononuclear leukocytes on discontinuous density gradients of Ficoll-Hypaque. *J. Immunol. Meth.* 5(3): 249-252
2. Tomlinson MJ, Tomlinson S, Yang XB, Kirkham J (2012). Cell separation: Terminology and practical considerations. *J. Tissue Eng.* 4: 2041731412472690.
3. Bonner WA, Hulett HR, Sweet RG, Herzenberg LA (1972) Fluorescence Activated Cell Sorting. *Rev. Sci. Instrum.* 43(3): 404-409
4. Miltenyi S, Muller W, Weichel W, Radbruch A (1990) High Gradient Magnetic Cell Separation With MACS. *Cytometry* 11(2): 231-238
5. Emad A, Drouin R (2014) Evaluation of the impact of density gradient centrifugation on fetal cell loss during enrichment from maternal peripheral blood. *Prenatal Diagnosis* 34(9): 878-885
6. Hartrich LA, Podniesinski EP, Stewart CC (1996) High Speed Cell Sorting-Determination of Post-Sort Viability, Yield and Purity. 18th Congress of the International Society for Analytical Cytology
7. Pohl HA (1951) The Motion and Precipitation of Suspensoids in Divergent Electric Fields. *J. Appl. Phys.* 22(7) 869-871
8. Pohl HA & Hawk IL. Separation of living and dead cells by dielectrophoresis. *Science* 152(3722): 647-649 (1966).
9. Markx GH, Huang Y, Zhou XF, Pethig R (1994) Dielectrophoretic characterization and separation of micro-organisms. *Microbiol.* 140(3) 585-591
10. Markx GH, Pethig R (1995) Dielectrophoretic separation of cells; continuous separation. *Biotechnol. Bioeng.* 45(4) 337-343

11. Stephens M, Talary MS, Pethig R, Burnett AK, Mills KI (1996) The dielectrophoresis enrichment of C34+ cells from peripheral blood stem cell harvests. *Bone marrow Transplantation* 18(4): 777-782
12. Talary MS, Mills KI, Hoy T, Burnett AK, Pethig R (1995) Dielectrophoretic separation and enrichment of CD34_ cell subpopulation from bone marrow and peripheral blood stem cells. *Med. Biol. Eng. Comput.* 33(2): 237-237
13. Becker FF, Wang XB, Huang Y, Pethig R, Vykoukal J, Gascoyne PR. Separation of human breast cancer cells from blood by differential dielectric affinity. *Proc. Natl. Acad. Sci. U S A.* 1995 Jan 31; 92(3): 860–864.
14. Fiedler S, Shirley SG, Schnelle T, Fuhr G (1998) Dielectrophoretic sorting of particles and cells in a microsystem. *Anal. Chem.* 70(9): 1909-1915.
15. Muller T, Schnelle T, Gradl G, Shirley SG, Guhr G (2000) Microdevice for cell and particle separation using dielectrophoretic field-flow fractionation. *J Liq Chrom Relat Tech* 23(1): 47-59
16. Cheng J, Sheldon EL, Wu L, Uribe A, Gerrue LO, Carrino J, Heller MJ, O’Connell JP (1998) Preparation and hybridization analysis of DNA/RNA from E-coli on microfabricated bioelectronic chips. *Nature Biotechnol.* 16(6): 541-546
17. Yang T, Huang Y, Wang X, Wang X-B, Becker FF, Gascoyne PRC (1999) Dielectric properties of human leukocyte subpopulations determined by electrorotation as a cell separation criterion. *Biophys. J.* 76(6): 3307-3314
18. H Morgan, MP Hughes, NG Green (1999) Separation of submicron bioparticles by dielectrophoresis. *Biophys. J.* 77(1): 516-525
19. HO Fatoyinbo, S Martin, P Pashby, MP Hughes, FH Labeed (2007) Dielectrophoretic Separation of Bacillus Subtilis spores from environmental diesel particles. *J. Env. Monit.* 9(1): 87-90

20. Pethig, R. (2017. Review—Where Is Dielectrophoresis (DEP) Going?. *J. Electrochem. Soc.*, 164(5): B3049-B3055.
21. Gupta V, Jafferji I, Garza M, Melnikova VO, Hasegawa DK, Pethig R, Davis DW (2012) ApoStream™, a new dielectrophoretic device for antibody independent isolation and recovery of viable cancer cells from blood. *Biomicrofluidics*. 6(2): 024133.
22. Hu X, Bessette PH, Qian J, Meinhardt CD, Daugherty PS, Soh HT (2005) Marker-specific sorting of rare cells using dielectrophoresis. *Proc. Nat. Acad. Sci. USA*. 102(44): 15757-15761.
23. Lee D, Hwang B, Choi Y, Kim B (2016) A novel dielectrophoresis activated cell sorter (DACS) to evaluate the apoptotic rate of K562 cells treated with arsenic trioxide (As₂O₃). *Sens. Actuat. A*, 242: 1–8
24. Markx GH, Talary MS, Pethig R (1994) Separation of viable and non-viable yeast using dielectrophoresis. *J. Biotechnol.* 32(1): 29-37
25. Fatoyinbo HO, Kamchis D, Whattingham R, Ogin SL, Hughes MP (2005) A high-throughput 3D composite dielectrophoretic separator *IEEE Trans. Biomed. Eng.* 52(70): 1347-1349
26. MA Abdul Razak, KF Hoettges, Fatoyinbo HO, Labeed FH, Hughes MP (2013) Efficient dielectrophoretic cell enrichment using a DEP-well based system *Biomicrofluidics* 7(6): 064110 (1-10)
27. Simon MG, Li Y, Arulmoli J, McDonnell LP, Akil A, Nourse JL, Lee AP, Flanagan LA (2014). Increasing label-free stem cell sorting capacity to reach transplantation-scale throughput. *Biomicrofluidics* 8(6): 064106, 2014
28. Labeed FH, Lu JT, Mulhall HJ, Marachenko SA, Hoettges KF, Estrada LS, Lee AP, Hughes MP, Flanagan L (2011) Biophysical characteristics reveal neural stem cell differentiation potential *PLoS One* 6(9): e25458

29. Vykoukal J, Vykoukal DM, Freyberg S, Alt EU, Gascoyne PR. (2008) Enrichment of putative stem cells from adipose tissue using dielectrophoretic field-flow fractionation. *Lab Chip* 8(8): 1386-93
30. Hughes MP (2016) Fifty years of dielectrophoretic cell separation technology. *Biomicrofluidics* 10(3): 032701
31. Duncan L, Shelmerdine H, Hughes MP, Coley HM, Hübner Y, Labeed FH (2008) Dielectrophoretic analysis of changes in cytoplasmic ion levels due to ion channel blocker action reveals underlying differences between drug-sensitive and multidrug-resistance leukaemic cells. *Phys. Med. Biol.* 53(2): N1-N7

Figure Legends

Figure 1. DEP spectra of two arbitrary cell types, whose properties are identical save for one (blue line) having a membrane capacitance twice that of the other (red line). In the frequency band (highlighted), the polarisabilities (and hence direction of force) of the two cells is different, enabling the cells to be separated.

Figure 2 (a). A photograph of the DEP separation chip. The chip is 30mm x 20mm in size. The inset section (red) shows a close-up of the chip, showing the electrodes along the inside of the wells. The section of the chip in the image is approximately 5mm square. (b) A schematic of chip, showing the three modes of DEP behaviour. Cells flow from top to bottom through the wells; in the left well cells can be seen experiencing positive DEP, are attracted to the electrodes and held; in the center well cells experience negative DEP, and are repelled into the centre of the well and pass through. In the third well, cells experience no DEP force. In reality, cells of the same type will experience the same mode of behaviour in all wells on the chip, but two different cell populations can exhibit different behaviors to each

other. If one subpopulation experiences positive DEP and the others exhibit negative or neutral behaviour, they can be separated. (c) The chip is loaded into a fluidic cartridge comprising two parts; an upper part B contains both housing and plunger, whilst a lower part A collects the cell solution. The chip fits between the two, sealed on both sides by O-rings, and is clamped together by three Allen bolts.

Figure 3. The average (n=3) results of the enrichment of mixtures of (a) live and dead yeast and (b) RBCs and fibroblasts, showing the total cell number in each case. “Initial” refers to the numbers at the start of separation, “Passed” represents the cells collected during the separation, “Recovered” represents those cells collected by positive DEP and subsequently removed, and “Lost” is those unaccounted for. Cells were processed through the device at approximately 1 million cells in 1 ml per minute.

Figure 4. The average (n=3) results of a second stage of separation of mixtures of (a) live and dead yeast and (b) RBCs and fibroblasts, showing the total cell number in each case. For the second step, the cell population experiencing negative dielectrophoresis was reprocessed. “Initial” refers to the numbers at the start of separation, “Outlet” represents the cells collected during the separation, “Waste” represents those cells collected by positive DEP and subsequently removed, and “Lost” is those unaccounted for. Cells were processed through the device at 1 ml per minute; with an initial cell concentration of approximately 1 million cells ml⁻¹.

Figure 5. Enrichment of MDA cancer cells from RBCs, at an initial ratio of 1:99, average (n=3) results using a three-pass protocol. (a) The percentage of RBC cells in the sample, per run. (b) The mean (n=3) overall recovery of the two cell types after each stage; the recovery of RBCs reaches a 99.1% suggesting that the cancer cells have been significantly enriched.

Figure 6. A schematic showing the separation procedure, which is divided into three steps. In step 1, a mixture of cells is drawn into the syringe, through the chip. In step 2, the chip is activated and cells are expelled at typically 1 ml min^{-1} . One cell type is retained in the electrodes whilst the other is eluted. In step 3, after the solution is fully expelled, fresh medium is drawn into the device and expelled manually at a higher rate, whilst the retaining field is deactivated, allowing the retained cells to be collected.

Figure 1

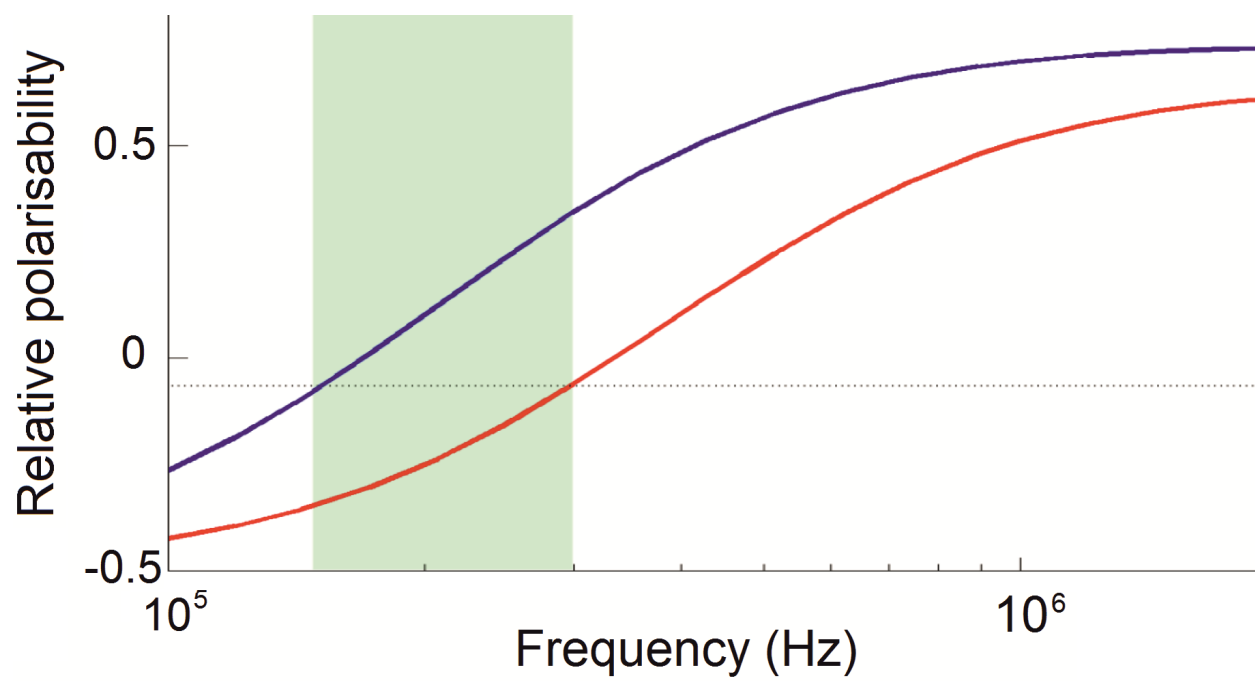


Figure 2

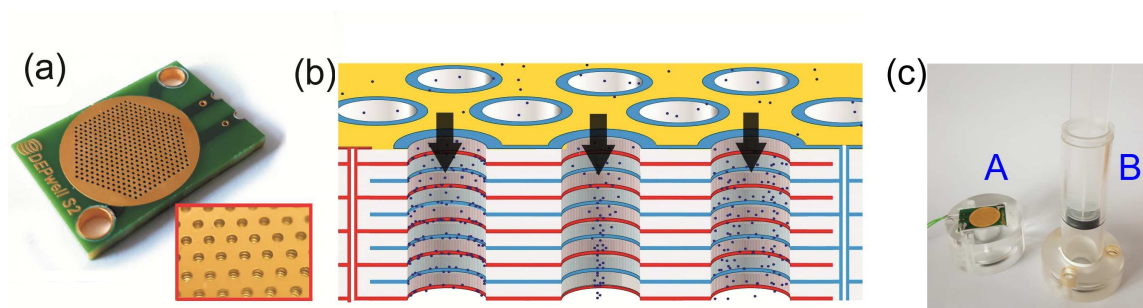


Figure 3

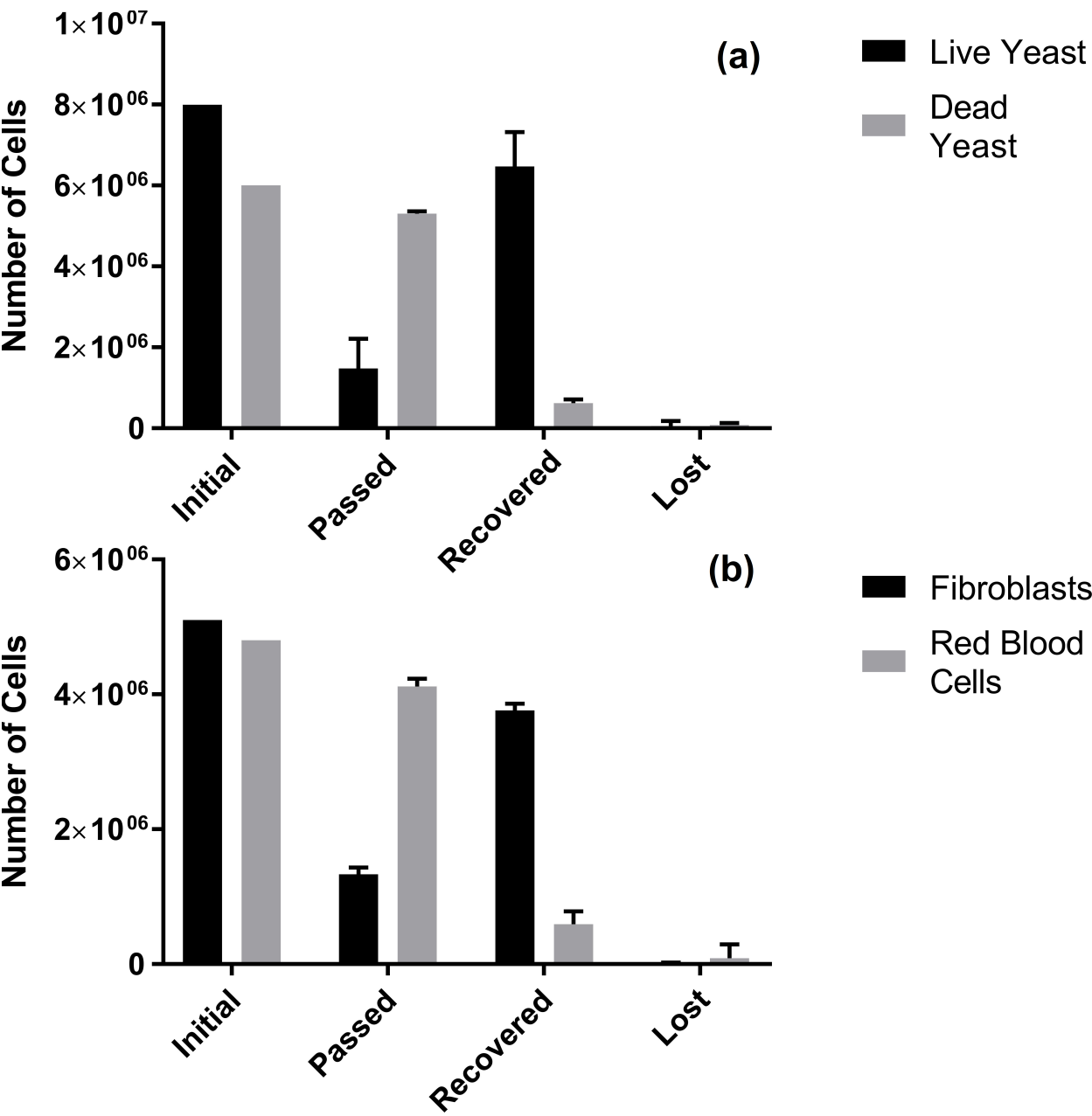


Figure 4

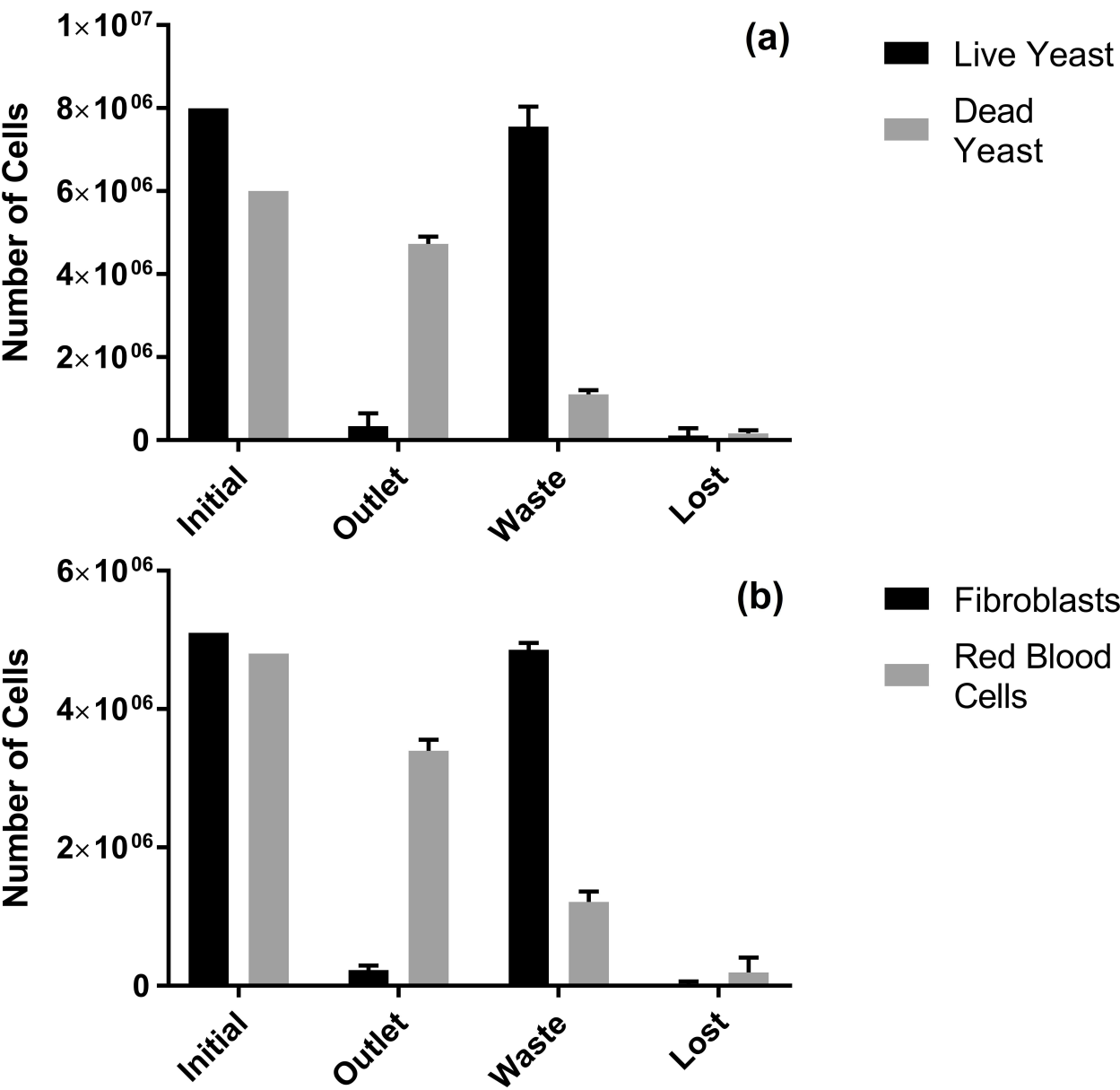


Figure 5

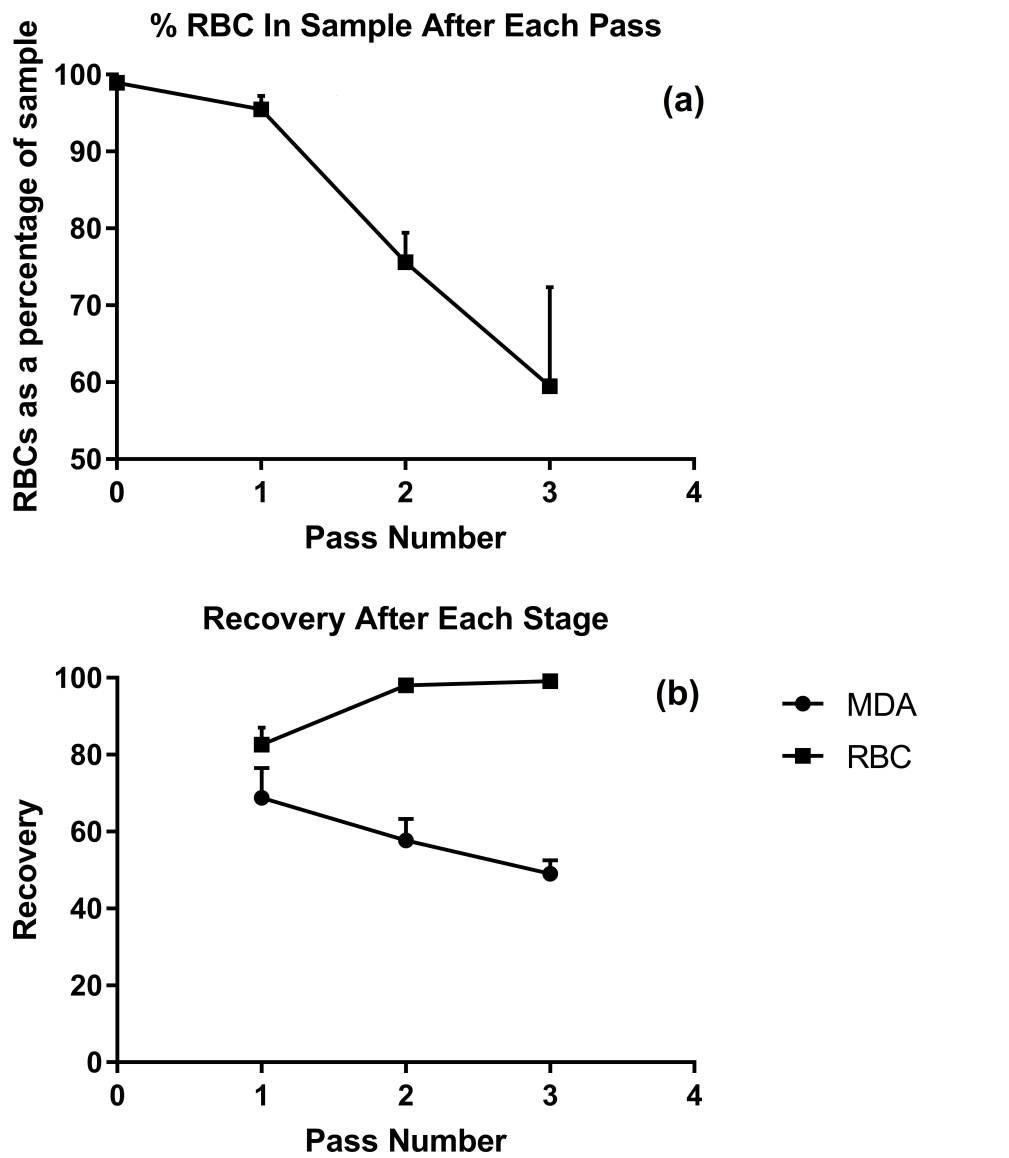
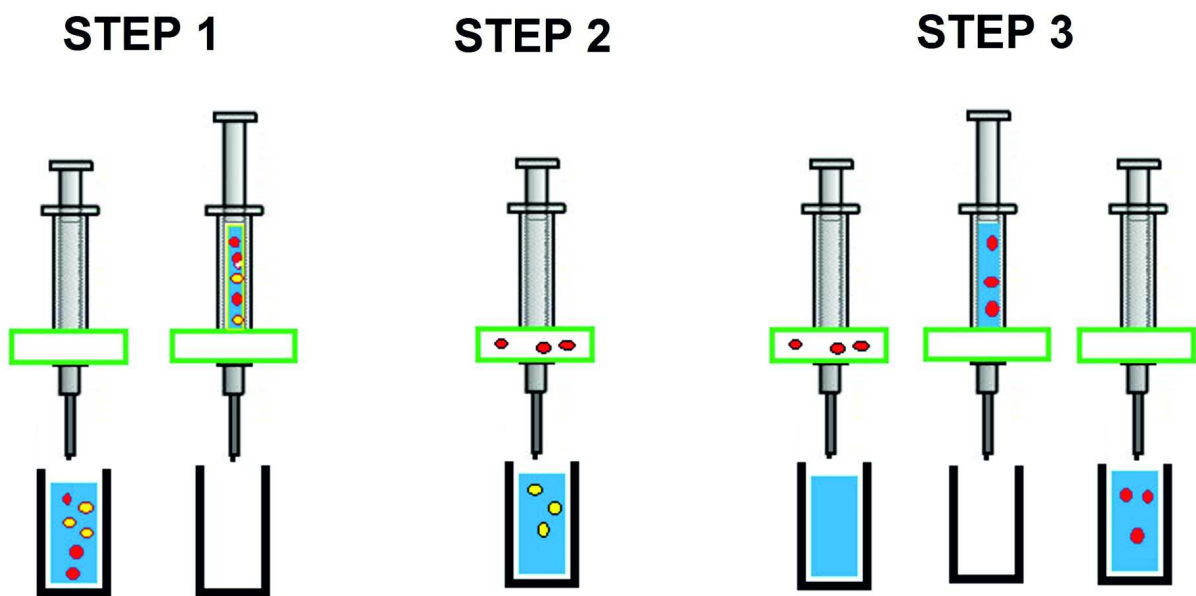


Figure 6



Cells

Live and dead yeast. Yeast was grown using Tesco's freeze dried baker's yeast grown in a yeast extract peptone dextrose (YPD) medium (Sigma-Aldrich, U.K.) set overnight in an incubator at 37 °C. Yeast cells were killed by transferring live yeast in YPD medium to an incubator set to 70 °C for 3 hrs. Yeast was washed and suspended in DI water before use in experiments; conductivity was not fixed, but was at or below 1 mSm⁻¹ (typically 0.2 - 0.5 mSm⁻¹).

Rodent Fibroblasts and Red Blood Cells. 3T3 mouse fibroblasts, kindly donated by Dr Andrew Liu, University of Memphis, U.S.A., were cultured in an incubator set at 37 °C and 5% CO₂ in growth medium. Cells were cultured in DMEM (Sigma-Aldrich, U.K.) with the addition of 10% Fetal Bovine Serum (Fisher Scientific), 1mM sodium pyruvate (Sigma-Aldrich, U.K.), 1% penicillin-streptomycin (Sigma-Aldrich, U.K.). Adult common voles were sourced from the University of Surrey's breeding colony in the UK, which was based on animals kindly donated by Professor Gerkema, University of Groningen, The Netherlands (S1-S3). Whole vole blood (1ml) was collected following published protocols (S4) using cardiac puncture, and collected in heparin coated tubes. Collection was followed by RBC isolation through three identical centrifugation steps (10 minutes, 1620g, 4°C), each followed by resuspension of the RBC containing pellet in Phosphate Buffered Saline. Finally, the two cell types were mixed and resuspended in DEP medium comprising de-ionised water supplemented with 8.5/0.3 % sucrose/dextrose (Sigma-Aldrich, U.K.), which were then washed and resuspended in DEP medium. The medium had a similar conductivity to the DI water sample described above.

Human cancer and red blood cells. Human blood was collected from donors at the University of Surrey and RBCs were isolated following published protocols (S5). The RBCs which were

then washed twice in DEP medium consisting of 8.5% (w/v) sucrose, 0.5% (w/v) dextrose, 100 μ M CaCl₂ and 250 μ M MgCl₂. MDA-MB-231 cells were culture in MEM medium (Sigma-Aldrich, UK) supplemented with 10% heat inactivated foetal bovine serum (FBS; Invitrogen, UK), 2mM L-Glutamine and 1% penicillin-streptomycin (Sigma-Aldrich, UK). The cells were grown in T75 flasks in a standard cell culture incubator at 5% CO₂ 95% humidified air at 37 °C and subcultured every 48 h when 80% confluent. To prepare for experiment, MDA-MB-231 cells were washed twice in a medium consisting of 8.5% (w/v) sucrose, 0.5% (w/v) dextrose, 100 μ M CaCl₂ and 250 μ M MgCl₂ and supplemented with NaOH to pH of 7.4. The medium conductivity was adjusted using PBS to 10 mS m⁻¹.

Preparation of Mixtures. Cell samples were prepared by counting using NanoEnTek C-chip disposable haemocytometer (Labtech, Uckfield UK), mixing to obtain an approximately 50:50 ratio, then re-measured. The stock of live and dead yeast was found to contain 8.0 million live cells and 6.0 million dead cells, which was processed as 15 ml samples (a concentration of 0.93 million cells ml⁻¹). Vole fibroblasts and RBCs were mixed, and the resultant mixture was measured to contain 5.1 million fibroblasts and 4.8 million RBCs in each of three 5 ml aliquots, a total concentration of 1.98 million cells ml⁻¹. A 20ml sample of 11.1million fibroblasts and 13.4 million RBCs was also prepared. Human red blood cells were spiked with MDA-MB-231 breast cancer cells, to a final concentration of 1.1% cancer to 98.9% RBCs, at a total cell concentration of ca. 2×10^7 ml⁻¹, to a total sample volume of 4ml.

Device Fabrication

The device comprised three principal parts; a fluidic chip, a casing, and the support instrument (including principally a signal generator, amplifier, and syringe driver). The chip comprised ten layers of 70 μ m-thick copper separated by 150 μ m-thick glass fibre reinforced

epoxy (FR4) layers, with two further 35 μm -thick copper layers at the top and bottom. All copper layers were patterned in a circle slightly larger than the area through which the separation wells were drilled, to minimise overlap and associated capacitive losses. 397 wells with a 400 μm -diameter were drilled in a hexagonal pattern at the centre of the chip; larger holes for assembly and contacts were placed at the perimeter of the chip. Exposed copper areas were gold-plated to maintain stability and biocompatibility. The chip was housed in a cartridge machined from PMMA, which comprised a reservoir containing a syringe plunger from a 20ml syringe, a chip holder, and an outlet. The chip formed the base of the reservoir, such that when the plunger was pushed, cells were able to flow through all 397 holes. Below the chip, a collection cone funnelled the suspension into a 1.46mm diameter outlet tube to a collection receptacle. The chip was mounted between top cylinder and bottom cone of the assembly and secured using Allen bolts, with a watertight seal provided by two 19.13mm-diameter O-rings.

References

- S1. van der Veen DR1, Saaltink DJ, Gerkema MP (2011) Behavioral responses to combinations of timed light, food availability, and ultradian rhythms in the common vole (*Microtus arvalis*). *Chronobiol Int.* 28(7):563-71
- S2. van der Veen DR1, Minh NL, Gos P, Arneric M, Gerkema MP, Schibler U (2006) Impact of behavior on central and peripheral circadian clocks in the common vole *Microtus arvalis*, a mammal with ultradian rhythms. *Proc Natl Acad Sci U S A.* 103(9):3393-8
- S3. van der Veen DR1, van der Pol-Meijer MM, Jansen K, Smeets M, van der Zee EA, Gerkema MP (2008) Circadian rhythms of C-FOS expression in the suprachiasmatic nuclei of the common vole (*Microtus arvalis*). *Chronobiol Int.* 25(4):481-99

- S4. Hanson MS, Stephenson AH, Bowles EA et al (2008). Phosphodiesterase 3 is present in rabbit and human erythrocytes and its inhibition potentiates iloprost-induced increases in cAMP. *Am. J. Physiol.* 295(2), H786–H793.
- S5. O'Neill JS, Reddy AB (2011). Circadian clocks in human red blood cells. *Nature*. 469, 498-503